INTRODUCTION

Recently, world cancer statistics showed that the incidence of breast and cervical cancer are globally on the rise [1]. Cervical cancer was the second highest diagnosed and the third leading cause of cancer death in females in ASEAN countries. It accounted for 11% of total new cancer cases and 9% the total cancer deaths among female in 2008 respectively [2]. Since cervical cancer also the most leading cause of female death in Indonesia [3], research focus on cervical cancer is an interesting topic.

Cervical cancer is transmitted by sexual intercourse that leads to human papillomavirus (HPV) infection. Oncogenic DNA is found in 95% of invasive HPV caused cervical cancers [4]. Previous studies showed that the E6 oncogenic protein from HPV form a complex with p53, a tumor suppressor gene and, as a result, targets it for rapid proteasome degradation [5,6]. Restoration of p53 function in cervical cancer is proposed to promote a selective therapeutic effect [6]. Moreover, reactivation of p53 is one of the key points in selective anticancer screening against this type of cancer.

Discovering selective anticancer agent against cervical cancer from plants is very challenging. Many compounds previously extracted from plant (vinblastine, vincristine, taxol) have excellent activity against several cancers. However, antioxidant and cytotoxic activity of an extract in inhibit cervical cancer cells since oxidative stress is one of inducing cervix carcinogenesis. Furthermore, the contribution of p53 reactivation in the molecular pathway of apoptosis was deeply observed through p53-expression levels in HeLa cells. This research was performed using HeLa cells, an in vitro model of cervical cancer that could express p53 under genetic stress condition.

MATERIALS AND METHODS

Materials
A. cordifolia leaf was cultivated and harvested from Wirobrajan, Yogyakarta. Dried A. cordifolia leaf was extracted using 70% ethanol (Merck) in the Phytochemistry Laboratory of Sanata Dharma University then the solvent was evaporated using a rotary evaporator and a dry extract was obtained by freeze drying. A HeLa cervical cancer cell line was derived in the Parasitology Laboratory, of the Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. It was cultured in Dulbecco’s Modified Eagle Media (DMEM) (Gibco) containing fetal bovine serum 10% (v/v) (Gibco) and penicillin-streptomycin 1% (v/v) (Gibco). Cytotoxic assay used 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) (Sigma) while the apoptosis kit used was Annexin V from Roche. Immunocytochemistry used p53 antibody and all tips were supplied by Biologix.

Methods

MTT cytotoxic assay
HeLa cells were seeded until confluent, then 5×10^4 cells plated into a 96-well microplate and incubated at 37°C and under 5% CO₂ for 24 hrs. The medium was removed and rinsed by phosphate-buffered saline (PBS) 10%. ALE and cisplatin were dissolved in dimethyl sulfoxide as stock.
The various concentrations of ALE in the medium were poured into 96-well plate at 100 µL each and incubated for 24 hrs. Each concentration was assayed in triplicates (n=3). The medium culture was removed and rinsed by PBS 10%, then 100 µL medium containing 5 mg/mL MTT was added into each well and incubated for 4 hrs. Further, the medium was removed and 100 µL SDS was added to each well to dissolve Formazan crystals. The 96-well microplate was incubated for 24 hrs in a dark room, avoid contact from light. Formazan crystals were measured by ELISA reader at a wavelength of 595 nm.

Apoptosis assay
HeLa was seeded 5x10^5/100 µL into coverslips in a 6-well plate, then acclimated at 37°C and under 5% CO2 for 24 hrs. They were resuspended in DMEM and placed in object glass. Later, they were fixed for 5 minutes and incubated in H2O2 for 10-15 minutes and then washed again using PBS. Monoclonal antibodies of p53 were added to the cells and incubated at least for an hour and washed three times using PBS. Secondary antibodies of biotinylated goat anti-polyvalent were dropped to the cells and incubated in room temperature for 10 minutes and washed four times in PBS. DAB as chromogen was dropped into the cells and incubated for 3-4 minutes, then washed using distilled water. Finally, hematoxylin solution was added and incubated for 3-4 minutes. These slides were dried and observed under a light microscope. Expression of p53 was indicated by brown cell color.

Analysis
The cytotoxic data of ALE were plotted and analyzed by excel then IC50 was linear regression using Excel MS Office 2010. Immucytostaining data of p53 were visual qualitatively analyzed by observing p53 expressing cells that showed brown color.

RESULTS AND DISCUSSION

Cytotoxic activity of ALE against cervical cancer has not yet been evaluated. In this study, cisplatin was used as positive control since it has been widely recommended to women with cervical cancer, especially combined with radiotherapy [10,11]. The drug strongly cross-links DNA, form intra- and inter-strand adducts that poorly repaired [12]. Even though cisplatin is an effective anticancer drug, it can cause severe side effect to many tissues namely that of peripheral nerves, renal tubules, the gastrointestinal tract, and bone marrow [13].

The MTT method demonstrated that ALE is a potential anticancer agent against cervical cancer cells with IC50 = 75 µg/mL (R²=0.9759) as shown in Fig. 1. Due to compounds complexity in ALE, cytotoxic activity of ALE was weaker than that of cisplatin, as pure compound with IC50 = 40 µM or equal to 12 µg/mL (R²=0.9908) (Fig. 2). But, both of ALE and cisplatin reduced HeLa cells viability in a dose-dependent manner and also manipulated the morphology of HeLa cells (Fig. 3). Cytotoxic activity of both of ALE and cisplatin could be related with a molecular mechanism induced apoptosis.

Apoptosis assay was determined using Annexin V that is able to bind phosphatidyserine residue in outer plasma membrane as the

![Fig. 1: Effect of Anredera cordifolia leaf ethanol extract on HeLa viability after 24 hrs incubation](image1)

![Fig. 2: Effect of cisplatin on HeLa viability after 24 hour incubation.](image2)

![Fig. 3: Effect of Anredera cordifolia leaf ethanol extract (ALE) and cisplatin on HeLa cells morphology after 24 hrs incubation](image3)
p53 and p73-related p53 protein were responsible in the apoptosis process of cisplatin in cervical cancer treatment [12]. Thus, p53 protein is interesting tumor suppressor gene to observe in relation to ALE cytotoxicity and apoptosis pathway.

Cervical cancer cells infected by an oncogenic virus are able to degrade tumor suppressor genes, like p53 by ubiquitination [5,6]. Since the restoration of p53 function promotes a selective therapeutic effect [6], this study focused on p53 role in apoptosis induction pathway of ALE against HeLa cells. ALE-treatment did not increase protein p53 expression in these cells (Fig. 6) compared to untreated cells. On the other hand, cisplatin treatment showed a slight increase in p53 expression. Cytotoxic activity of ALE was not related with p53 restoration in HeLa cells. The molecular mechanism of ALE anticancer activity was p53-independent pathway. Further, study on other proteins involved in this molecular mechanism of anticancer activity must be conducted.

CONCLUSION

This research concludes that Anredera cordifolia leaf extract is promising anticancer agent against HeLa cervical cancer cells with an IC50 value of 75 µg/mL. It definitely triggered apoptosis on HeLa cells through p5-3 independent pathway. Other regulating proteins involved in this molecular mechanism must be investigated.

ACKNOWLEDGMENT

This work was financially supported by Institute for Research and Public Affairs, Sanata Dharma University (Internal Research Grant No 081/Pen Dosen Muda/LPPM USD/VI/2014).

REFERENCES


